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The Reliability of a Mechanized Procedure (Perkin-Elmer C4) for the Enzymatic Determination of Uric Acid According to *Kageyama*

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Summary: The enzymatic determination of the uric acid concentration in urine and serum according to *Kageyama* ((1971), Clin. Chim. Acta *31*, 421–426), which excludes the deproteinization of samples, was adapted to the C4 automatic analyzer (Perkin-Elmer). The reliability of this procedure and its correlation with an UV-method were investigated. The interaction from-sample-to-sample was considerable, but this could be reduced by the addition of Brij-35.

The recovery of uric acid added to protein-containing samples (about 96%) was better than with the UV-method. Novaminsulfone was the only substance tested which interfered significantly.

Zuverlässigkeit eines mechanisierten Verfahrens (Perkin-Elmer C4) für die enzymatische Bestimmung von Harnsäure nach Kageyama

Zusammenfassung: Die enzymatische Bestimmung der Harnsäure Konzentration nach *Kageyama* ((1971), Clin. Chim. Acta *31*, 421–426) wurde an das Analysengerät C4 (Perkin-Elmer) adaptiert und deren Zuverlässigkeitskriterien untersucht. Die Verschleppung von Probe zu Probe war beträchtlich, konnte jedoch durch Zusatz von Brij reduziert werden.

Zusatzversuche von Harnsäure mit proteinhaltigen Proben ergab eine höhere Wiederfindung (um 96%) als mit einer vergleichenden UV-Methode. Bei einer Reihe von getesteten Substanzen führte nur Novaminsulfon zu einer signifikanten Interferenz.

Introduction

Kageyama reported recently (1) a new procedure for the direct enzymatic measurement of the uric acid concentration in urine and serum. This method uses the *Hantzsch*' reaction to determine formaldehyde formed from uric acid by means of the two enzymes uricase (EC 1.7.3.3) and catalase (EC 1.11.1.6). The resulting color (3,5-diacetyl-1,4-dihydrolutidine) has its absorbance maximum at 410 nm.

This procedure appeared to be suitable for the mechanization with a discrete analytical system. For this purpose we used the Perkin-Elmer C4 Automatic Analyzer, a combined-discrete system (2), since the reaction mixture is transported in single cups and then transferred into a discontinuous flow system for the photometric measurement.

In the following investigations on the reliability of the *Kageyama* method adjusted to the C4 analyzer are reported. The direct enzymatic UV-procedure of *Praetorius* (3) was chosen as the reference and performed manually because we did not succeed in adapting this method to the C4 analyzer with sufficient precision. A sample blank assay treated with a glycerol solution instead of the uricase suspension was used according to *Kortüm* (4).

Methods and Materials

Operation of the C4 Automatic Analyzer

The analyzer ist operated according to the instructions of Perkin-Elmer as shown in figure 1. The incubation time is 43 minutes at 37 °C. Sample batches are divided in 3 different segments as indicated in figure 2. The inter-segment B can be repeated as often

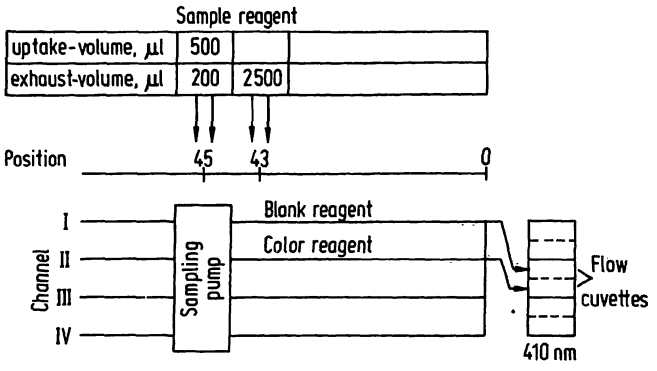

Fig. 1. Adaptation of the C4 Automatic Analyzer for the uric acid determination.

as required. The concentration factor is calculated from the first standard samples in segment A and introduced into the control unit. The printed values of the standard solutions in B and C segments are corrected directly with the factor potentiometer if necessary. Blanks (bidist. H_2O) can be omitted between 3 B segments. Urine samples (50 µl) are diluted with bidist. water (500 µl) using Eppendorf pipettes.

Solutions

1. Colour reagent:

$(NH_4)_2HPO_4$ (757 mmol/l	100 g	Merck No. 1207
H_3PO_4, 85% (ca 44 mmol/l)	3 ml	Merck No. 573
CH_3OH (ca. 2.47 mol/l)	100 ml	Merck No. 6009
Acetylacetone (19 mmol/l)	2 ml	Merck No. 9600
Catalase	100 mg	Boehringer No. 15674
Uricase	10 mg	Boehringer No. 15074
Brij-35	1 ml	Technicon No. 0214-15
Bidist. H_2O	ad 1000 ml	

2. Blank reagent: as solution (1) without uricase. Colour and blank reagent are filtered (Selecta filter No. 595 1/2, Schleicher and Schüll, D-3354 Dassel).

   Both solutions are stable for 10 days at 4 °C according to l.c. (1). The pH value of both colour and blank reagent is about 7.4 and is not adjusted to 7.0 as proposed by *Kageyama* (1).

3. Lithium carbonate, 135 mmol/l

Li_2CO_3	1.0 g	Merck No. 5671
Bidist. H_2O	to 100 ml	

4. Uric acid stock standard, 5 mmol/l. This solution is prepared according to *Liddle* et al. (5), chloroform is used as preservative (6).

Uric acid, dried	840.6 mg	Merck No. 814
Bidist. H_2O (60–80 °C)	500 ml	
Li_2CO_3 1 g/100 ml (60–80 °C)	50 ml	

   After the uric acid is dissolved, allow to cool to room temperature, then add

$CHCl_3$	1 ml	Merck No. 2431
Bidist. H_2O	to 1000 ml	

   This solution is stable for 1 month at 4 °C.

5. Uric acid working standard, 500 µmol/l:

Uric acid stock standard	10 ml
Li_2CO_3 1g/100 ml	5 ml
Bidist. H_2O	to 100 ml

   This solution is prepared each day.

Other materials were purchased from E. Merck AG (D-6100 Darmstadt), bovine albumin (purest, dried) from Behring AG (D-3550 Marburg) and Seronorm from Dr. Molter GmbH (D-6900 Heidelberg).

The uricaquant test (Boehringer Mannheim, No. 15865) was performed according to the instructions of the manufacturer.

For the direct enzymatic determination of the uric acid concentration at 293 nm the Boehringer test combination No. 15986 and a Zeiss PM 4 spectrophotometer were used. All results were referred to the working standard solution (5).

The *interaction* from low to high concentrations (Q_1) and from high to low concentrations (Q_2) was determined as recently reported (7) and expressed as the percent interaction coefficient ($Q = 100 \times q$; for the explanation of q see Fig. 2).

The *recovery* of uric acid added to various samples was calculated from the results of the following assays:

1. 9.0 ml Li_2CO_3 solution (500 mg/l) + 1.0 ml stock standard (5 mmol/l uric acid);
2. 9.0 ml serum + 1.0 ml stock standard; and
3. 9.0 ml serum + 1.0 ml Li_2CO_3 solution (500 mg/l).

Statistical evaluation of precision data were performed according to l.c. (10).

Results and Discussion

Under our test conditions, the absorbance of the reaction mixture slowly increased even in the absence of uric acid (fig. 3). Precision therefore depends upon exact

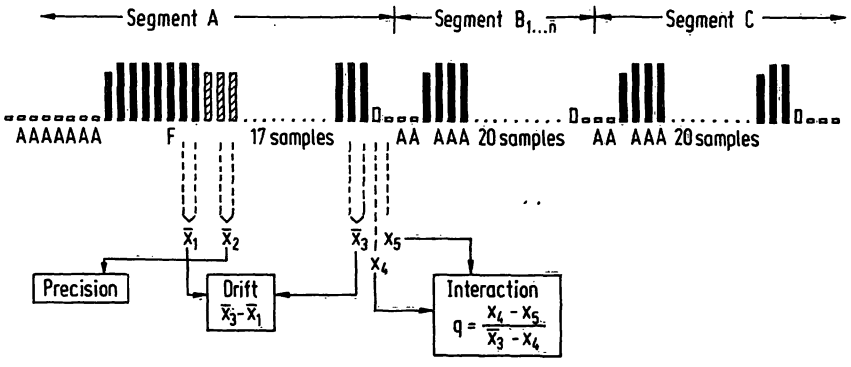

Fig. 2. Sequence of samples introduced into the C4 Automatic Analyzer for the uric acid determination.
A: adjustment of the corresponding potentiometer if necessary. At F the concentration factor is calculated from the 4 preceeding values and introduced into the control unit. ▭ Blank (H_2O) ■ Standard ▨ Control.

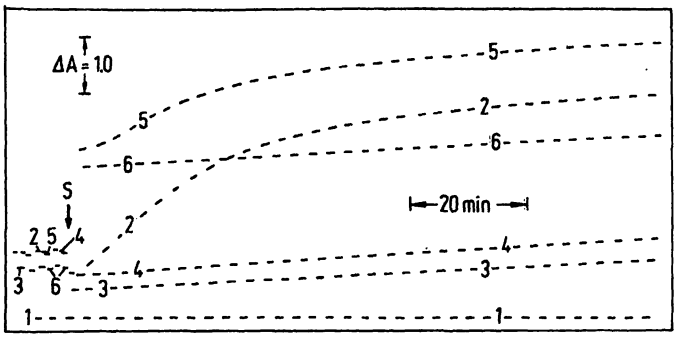

Fig. 3. The reaction rate of the uric acid determination according to *Kageyama* at 410 mm and 37°C (Zeiss photometer PM 4 with automatic cuvet changing device). Cuvet 1: picric acid solution for automatic correction of E = O. Cuvet 2: 2500 µl reaction solution (rs)+200 µl uric acid solution (500 µmol/l); cuvet 3: 2500 µl blank solution (bs)+200 µl uric acid solution (500 µmol/l); cuvet 4: 2500 µl rs + 200 µl bidist. H_2O; cuvet 5: 2500 µl rs + 200 µl Monitrol I; cuvet 6: 2500 µl bs + 200 µl Monitrol I. The reaction was started by adding the sample as indicated by the arrow.

timing. This can easily be achieved by using an analyzer which transfers the prepared samples to the photometer in the same time sequence as it starts the reaction.

Precision

The precision of the mechanized procedure is summarized in table 1. The requirements of the College of American Pathologists (precision from day to day: co-coefficient of variation < 4.6%) and the Guidelines of the Bundesärztekammer for Statistical Quality Control and Collaborative Surveys (precision from day to day: coefficent of variation < 10%) have been satisfied (9, 10).

Accuracy

The detection limit calculated according to *Kaiser* (8) is 18 µmol/l. The calibration curve is nearly linear up to a concentration of 1500 µmol/l (fig. 4). A sufficient correlation was found when the uric acid concentration of

Tab. 1. The precision of the enzymatic determination of uric acid with the C4 analyzer.

Sample	Precision within series			Precision from day-to-day		
	$\bar{x}$ [µmol/l]	s (n)	CV [%]	$\bar{x}$ [µmol/l]	s (n)	CV [%]
Standard solution	52.8	1.7 (20)	3.2			
	101.7	1.3 (20)	1.3			
	206.5	1.6 (20)	0.8			
	501.0	2.1 (20)	0.4			
	989.8	3.9 (20)	0.4			
Monitrol I	465.0	5.2 (19)	1.1	465.0	9.1 (19)	2.0
Seronorm	391.9	3.3 (10)	0.8	391.9	7.2 (10)	1.8

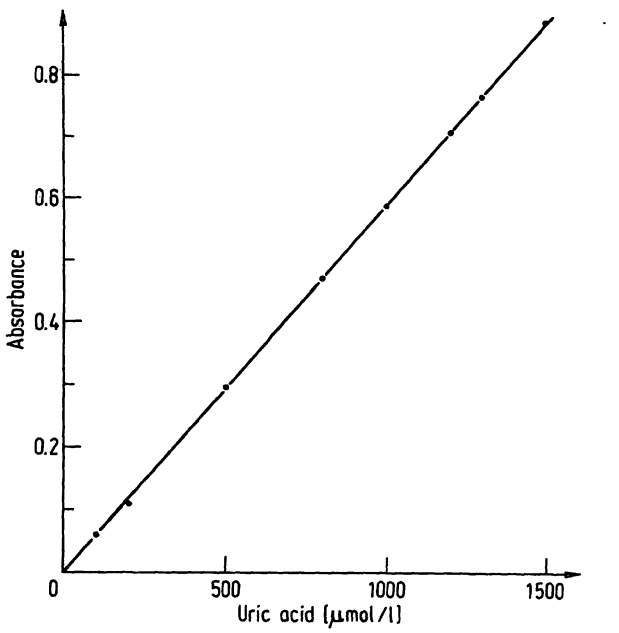

Fig. 4. Calibration curve of the uric acid determination according to *Kageyama* (1) Each point represents the mean of 5 values.

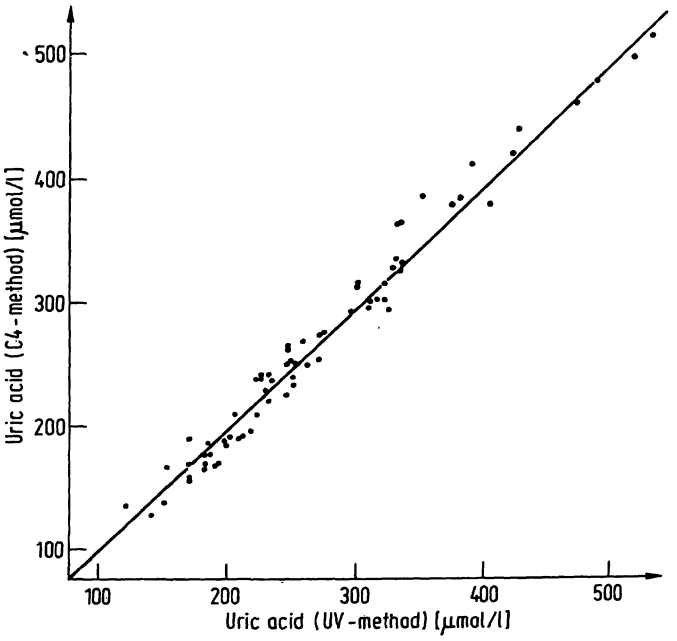

Fig. 5. The correlation of the uric acid concentration in serum samples from 70 patients determined according to *Kageyama* (1) and with an UV-method: y = 0.99 x + 0.56 (r = 0.99).

serum and urine samples from several patients were determined with this and with a direct UV-method (fig. 5 and 6). Recovery studies with pooled urine and serum samples are summarized in table 2. For recovery studies it appeared necessary to add albumin to the standard solution (70 g/l). Otherwise approximately 96% of the uric acid added were recovered (tab. 2). This effect was influenced by the protein (tab. 3) and the uric acid concentration (tab. 4); it was more pronounced with the UV-

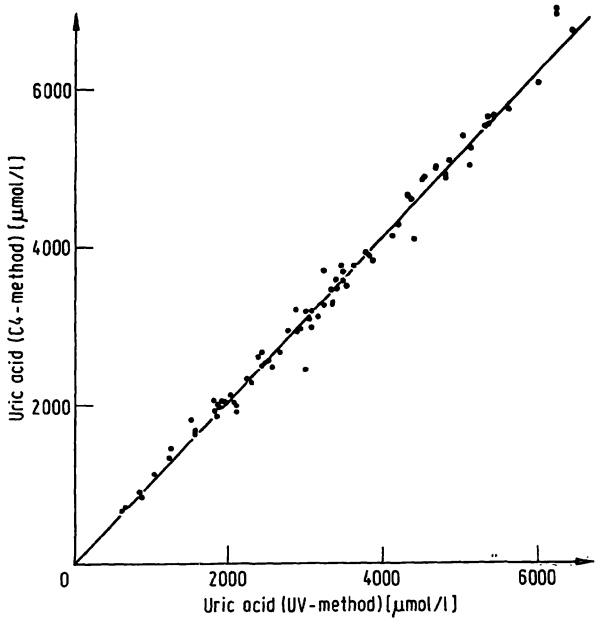

Fig. 6. The correlation of the uric acid concentration in urine samples from 80 patients determined according to *Kageyama* (1) and with an UV-method: y = 1.03 x + 5.59 (r = 0.99), n = 79.

Tab. 2. Recovery of uric acid added to various human serum and urine samples. Each value (µmol/l) is a mean of at least 2 determinations.

Sample	Uric acid added [µmol/l]	found [µmol/l]	Recovery [%]
A) Calibration with a standard solution containing 70 g/l albumin			
Serum	500	499	99.8
Serum	314	313	99.7
Serum	291	291	100.0
Serum	321	320	99.7
Serum	493	490	99.4
B) Calibration with a protein-free standard solution			
Urine	489	489	100.0
Urine	488	492	100.8
Urine	510	500	99.8
Serum	469	431	91.9
Serum	467	442	94.6
Serum	298	270	90.6
Serum	294	286	97.3
Serum	544	520	95.6
Serum	540	521	96.5
Serum	547	529	96.7
Serum	280	272	97.1
Serum	508	472	94.1
Serum	512	480	93.7
Pooled Serum	497	480	96.6[a])
albumin solution (70 g/l)	500	482	96.4[b])

[a]) mean value from various experiments at 12 days
[b]) n = 2.

Tab. 3. The influence of albumin on the uric acid concentration. Purified human albumin was added to a primary standard solution of uric acid (500 µmol/l). Each value (µmol/l) is a mean of 4 determinations.

Albumin concentration g/l	UV-method Zeiss photometer PM 4 Uric acid [µmol/l]	*Kageyama* procedure C4 analyzer	Eppendorf photometer 1101 (Uricaquant)
0	500	500	502
27	485	498	–
63	456	490	488
126	412	478	465

Tab. 4. The recovery of uric acid from aqueous solutions containing bovine albumin (135 g/l) in relation to the uric acid concentration. Each value (µmol/l) is a mean of 3 determinations.

Uric acid concentration (C4 analyzer) with albumin [µmol/l]	without albumin [µmol/l]	% of control (in the absence of albumin)
53	42	79
108	93	86
510	476	93
999	943	94

method (tab. 3) and can probably be explained by the urate binding capacity of albumin and other serum proteins (11, 12, 13). When primary standard solutions are used for the calibration the results only correspond to the unbound part of the total uric acid concentration in serum.

With uricaquant, a commercially available test combination for the *Kageyama* procedure, the same results were obtained if the test was performed either with a C4 analyzer or manually following the instructions of the manufacturer (tab. 3).

Serum samples can be stored in a refrigerator (+ 4°C) for at least one week. During this time the uric acid concentration is fairly stable (tab. 5).

Tab. 5. The stability of uric acid in human sera and urines if stored at 4°C.

Storage, days	0	1	2	3	4
	Uric acid [µmol/l]				
Serum No. 1	368	370	370	365	369
2	373	387	377	379	378
3	341	347	345	339	340
4	315	326	313	319	322
$\bar{x}$	349	358	351	351	352
	Uric acid [mmol/l]				
Urine No. 1	3.5	3.6	3.5	3.5	3.5
2	4.4	4.6	4.6	4.5	4.6
3	4.7	4.4	4.6	4.5	4.6
4	1.1	1.2	1.2	1.2	1.2
$\bar{x}$	3.43	3.45	3.48	3.43	3.48

Each value is the mean of 2 determinations

Drift effects

As recently pointed out (2) drift- and interaction effects must be carefully investigated with fully mechanized analytical methods. Baseline- and sensitivity drift (2) between 20 samples were determined in segment A and C (Fig. 2) of several batches on different days. The sensitivity drift was less than 1% (tab. 6), if measured with a concentration from the middle of the linear part of the calibration curve (2). The baseline drift need not be observed in every batch because it has the same value as the sensitivity drift (tab. 6).

Tab. 6. Drift effects in segment A and C (1 intersegment B). The numbers are means (µmol/l) from several determinations on different days. The calculation of the daily value occurred as shown in Fig. 2. The sensitivity drift was investigated with a standard containing 500 µmol/l uric acid, the baseline drift with bidist. H_2O.

Segment	A	C
Baseline drift	| 2.7 | (n = 17)	| 2.7 | (n = 17)
Sensitivity drift	| 2.8 | (n = 19)	| 2.9 | (n = 14)

Interaction effects

From a theoretical standpoint 2 types of interaction are possible with the C4 Automatic Analyzer. A cyclic interaction (2) could not be detected when investigated according to the recently reported method for the determination of chloride (7), using a solution with 2000 µmol/l uric acid and 7 g/100 ml purified albumin. The interaction from sample-to-sample was considerable and more pronounced in the presence of protein (tab. 7). The addition of Brij-35 reduced the carry-over significantly (tab. 7).

Several procedures have been proposed (2) for the correction of all results by means of an interaction coefficient determined at the beginning of each batch from a series of 3—4 interaction standards. The coefficient of interaction varies considerably, even if determined serially (tab. 7). Therefore, a mean value of several determinations is preferable for the correction of all batch results.

It is common practice to neglect carry-over effects if the data cannot be processed by a computer. Since the interaction from sample-to-sample of the mechanized procedure was considerable (tab. 7), it was necessary to determine whether the effect on the results was sufficient to warrant correction. For this purpose, we postulate that the difference between the true and the observed value shall be lower than the 3fold standard deviation of the corresponding mean value from several intrabatch determinations (tab. 1). Then, in extreme cases the result can differ approx. 3% from its true value in the normal range of the human serum concentration. Using a procedure recently described (7) the concentration range, in which interaction effects can be tolerated, was calculated (fig. 7). The analysis of a sample must be repeated if the preceding value is outside the so called interaction-safe range (fig. 7) or higher than 1000 µmol/l.

Tab. 7. The interaction from sample to sample in the enzymatic determination of uric acid with the C4 analyzer.

Protein content of the sample	Q_1 $\bar{x}$ (%), s (n)	Q_2 $\bar{x}$ (%), s (n)
no protein (variation within series)	2.4 ± 0.4 (10)	2.5 ± 0.4 (10)
no protein (variation from-day-to-day)	2.4 ± 1.0 (18)	2.5 ± 1.0 (18)
albumin 70 g/l (variation from day-to-day	3.8 ± 1.3 (12)	4.5 ± 1.7 (12)
albumin (70 g/l) and Brij 35 (1 ml/l) (variation from day-to-day)	2.5 ± 0.4 (15)	2.4 ± 0.4 (15)

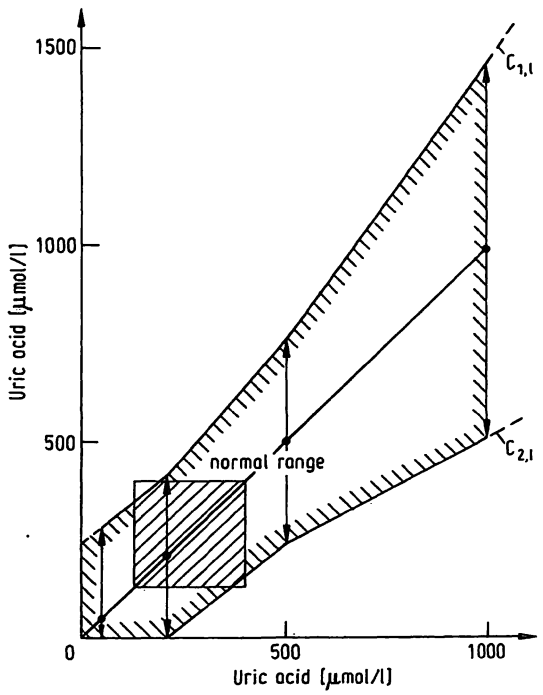

Fig. 7. Concentration range in which interaction effects can be tolerated with the C4 Automatic Analyzer.
The concentrations forming the interaction-safe range are calculated according to (7): C1, *l* for the interaction from low to high concentrations and C2, *l* for the interaction from high to low concentrations. The standard deviations are taken from table 1, the Q values from table 4 (last line).

Tab. 8. The influence of ascorbate and bilirubin on the determination of the uric acid concentration according to *Kageyama* (1).

Substance	Uric acid added [µmol/l][1])	found	Recovery [%]
Na-ascorbate 0.5 g/l	500.5	499.0	99.7
Na-ascorbate 1.0 g/l	500.5	496.5	99.2
Bilirubin	500.5	502.0	100.3

[1]) mean value of 2 determinations

Interferences

Interference from bilirubin, triglycerides and ascorbate can be neglected with the determination of uric acid according to *Kageyama* (1) under the present conditions (tab. 8). Further studies were undertaken with a series of drugs representing the most common therapeutics used in human medicine (tab. 9). This list, in which the trade name mentioned may be considered as an example for all other drugs containing the same substance, was recommended by *Staehler* et al. (14). All substances were added in 10 ml 9 g/l NaCl to 40 ml of the same pool-serum. These samples were analyzed in various series together with several control samples (10 ml 9 g/l NaCl + 40 ml pool-serum). In the presence of tetracyclinum and methyldopum the uric acid concentration was determined slightly below the 2s-range (tab. 8); this effect could not be confirmed in further experiments. Novaminsulfone was the only substance which caused a consistent underestimation of the uric acid concentration (tab. 10). The oral intake of 6 mg/kg novaminsulfone ($^1/_8$ of the maximal dosage per day) leads to a serum concentration of 14 mg/l (16).

Tab. 9. Recovery of uric acid in human pooled sera containing various drugs. In the absence of any substance added a mean value of 239 µmol/l uric acid was found (n = 43, s = 10.91, 2s – range = 217–261).

Trade name	I. N. N.[a])	concentration [mg/l]	uric acid [µmol/l]
Glifanan	glafeninum	240	222
Aspirin	acidum acetylosalicylicum	600	243
Butazolidin	phenylbutazonum	120	250
Novalgin	novaminsulfonum	800	140[b])
Buscopan	hyoscin-N-butylbrominum	12	243
Amuno	indometacinum	30	221
Dolviran	acidum acetylosalicylicum, etc.	480	251
Prolixan 300	azopropazon-dihydrat	360	241
Actol	acidum niflumínicum	150	232
Tanderil	oxyphenbutazonum	120	257
Metalcaptase	*D*-penicillaminum	480	234
Zyloric	allopurinolum	80	251
Uricovac	benzbromaronum	20	259
Benemid	probenecidum	200	230
Lanicor	digoxinum	0.15	217
Intensain	carbocromenum	90	237
Novadral	norfenefrinum	6	245
Miroton	glycosides, etc.	6 ml/l	248
Aldaktone	spirolactonum	20	222
Sembrina	α-methyldopum	320	208[b])
Modenol	thiabutazide, etc.	2.6	254
Dipar	phenylethylbiguanide	30	238
Euglycon	glibenclamidum	3	234
Rastinon	tolbutamidum	400	240
Solu-Decortin	prednisolonum	200	233
Aponal	doxepinum	30	228
Librium	chlordiazepoxidum	20	228
Methotrexat	acidum methylpteroyl-glutaminicum	1	237
Endoxan	cyclophosphamidum	40	227
Megaphen	phenothiazinum	30	217
Luminal	acidum phenylaethylbarbituricum	80	220
Hostacyclin	tetracyclinum	200	212[b])
Paraxin	chloramphenicolum	600	217[b])
Binotal	aminobenzylpenicillinum	600	240
Sulfa-Furadantin	sulfametum	300	237
Furadantin	nitrofurantoinum	30	229
Durenat	sulfanilamidopyrimidinum	200	236
Refobacin	gentamycinum	6	242
Lasix	furosemidum	20	221
Dulcolax	bisacodylum	4	244
Angiografin	acidum trijodbenzoicum	4 ml/l	253
Urografin	acidum trijodbenzoicum	4 ml/l	246
Biligrafin	adipinyltrijodanilidum	4 ml/l	223
Resochin	chloroquinum	100	243
Polybion	vitamine B complex	0.8 ml/l	241
Nicobion	nicotinamidum	40	246
Cebion	acidum ascorbicum	400	229
Marcumar	phenprocoumonum	6	236
Macrodex	dextranum 6%	100 ml/l	246
Neoplasmagel	gelatine 6%	100 ml/l	247
Anticoagulantia	Na-oxalate	3000	251
	Na-fluoride	2000	256
	Titriplex III	1000	253
	Na-heparinat	750	248
	Na-citrate	5000	238
Dura-Clofibrat	clofibratum	400	217
Antistin	antazolinum	160	234

[a]) international non-proprietary names as proposed by the WHO (15).
[b]) this value is outside the 2s-range.

Tab. 10. The effect of novaminsulfone on the uric acid concentration determined according to *Kageyama* in aqueous solutions.

novaminsulfone [mg/l]	uric acid[a]) [µmol/l]	% of control (in the absence of novaminsulfone)
A) aqueous solutions		
0	500	–
10	491	98
20	482	96
100	431	86
800	352	70
B) pooled serum		
0	388	–
400	255	66
800	229	59

[a]) mean values of 2 determinations

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